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BIOLOGICAL DELIGNIFICATION OF WOOD AND STRAW FOR ETHANOL PRODUCTION via SOLID STATE CULTURE

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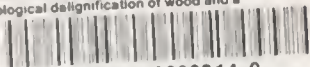
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FINAL REPORT

DNRC GRANT AGREEMENT NO. RAE-86-1066

Biological Delignification of Wood and Straw
for Ethanol Production
via Solid State Culture

Submitted to:

Montana Department of Natural Resources and Conservation
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November 15, 1989

NOTICE

This project was undertaken in part with government support under contract no. DE-FG79-82BP35776 awarded by the Bonneville Power Administration and a grant awarded by the Montana Department of Natural Resources and Conservation. Such support does not constitute an endorsement by BPA or DNRC of the views expressed in this work, and the user agrees to hold BPA and DNRC harmless against any direct or consequential damages claimed by the user or their parties arising from or related to use or interpretations of this report.

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INTRODUCTION

Lignocellulosic materials are a promising feedstock for alcohol fuel production. Lignocellulosic materials are abundant and renewable. As an alternative energy source, low-value or waste lignocellulosic material can be used without affecting supplies of food and fiber.

Millions of tons of low-value or waste lignocellulosic material are available in Montana as byproducts of the agriculture and forest products industries. Much of this material is discarded, which creates solid waste disposal problems. With efficient low-cost conversion technology, wastes which are now an economic liability can be turned into an asset as an energy source.

Lignin, a complex polymer of phenylpropanoid molecules in nonlinear random linkages, is the major structural component of plants. Cellulose is encased in a lignin matrix which must be degraded before the cellulose can be enzymatically hydrolyzed to fermentable sugars.

Most research in lignocellulose conversion has concentrated on mechanical and chemical pretreatments to remove lignin and has emphasized enzymatic hydrolysis of cellulose fractions. Pretreatments often involve heat and are capital and energy intensive. Chemical solvent extractions also have a potentially adverse environmental impact.

A report by the Solar Energy Research Institute¹ contains a detailed description and economic analysis of lignocellulose mechanical/chemical pretreatment followed by enzymatic hydrolysis and fermentation of the cellulose. Two of the most limiting economic factors in alcohol production from lignocellulose are pretreatment and cellulase costs.

In research supported by the U.S. Department of Energy under the Small Business Innovation Research Program (SBIR), Mycotech adapted solid state culture (SSC) technology to production of a low-cost cellulase for use in biomass conversion.² This work successfully developed a cellulase preparation that is about one-fifth the cost of liquid culture preparations for equivalent activity levels. The potential to use Mycotech's SSC technology to produce lignin degrading enzymes led to the concept of a completely biological process for producing alcohol from lignocellulosic feedstocks.

White rot fungi are a class of wood decaying organisms that enzymatically degrade lignin.³ One organism, Phanerochaete chrysosporium has been the subject of considerable research.⁴ The lignin degrading enzyme system produced by this organism in nitrogen limited, stationary liquid culture systems has been purified.⁵ White rot fungi have high oxygen requirements^{6,7,8} and unusual responses to nitrogen and carbon concentration in liquid growth media.^{9,10} As a result it has been difficult to develop efficient liquid culture systems that can be scaled up

economically for ligninase* production or lignocellulose pretreatment. White rot fungi produce ligninases when grown on solid substrates; however, commercial application is seen as infeasible because of long culture times and because solid culture technology is generally regarded by the U.S. fermentation industry as unconventional and difficult to apply on a large scale.

With support from DNRC¹¹ and USDOE,² Mycotech developed innovative solid culture technology that is:

- low in capital and operating cost
- suitable for large scale operations
- widely adaptable to fungi that perform poorly in liquid culture

This technology has been developed on a small scale for a pilot project to produce amylase and cellulase enzyme preparations. It is based on innovations in substrate characteristics, culture reactor design, and monitoring and control systems. Computerized monitoring and control systems were developed with support from DNRC.¹¹

Mycotech's proposal to develop a process for biological delignification arose out of the company's SSC capability, the development of a low-cost cellulase, and the availability of sophisticated SSC monitoring and control systems. The proposal was submitted in March 1986, and the grant was awarded June 1986.

* The term ligninase will be used throughout this report to refer to the multiple enzymes involved in degradation or modification of lignin.

During this project, two other contracts were obtained for work with white rot fungi. The first was a U.S. Department of Energy phase I SBIR project to evaluate solid culture for production of cell-free ligninase enzyme preparations. The second was a contract with a private European company to produce ligninase preparations from a variety of white rot fungi for application testing in improving paper pulp. These projects provided a much wider range of enzyme samples for analysis in the DNRC project than would otherwise have been available.

RESEARCH METHODOLOGY

The overall objective of the project was to find a process for biological delignification that could be integrated with SSC technology to produce and use a low-cost cellulase on Montana-specific lignocellulosic materials to produce ethanol. Meeting this objective would result in a low-cost biological technology which integrates lignin removal, cellulose hydrolysis, and fermentation to convert lignocellulose to alcohol fuel. Work emphasized the use of low-value or waste material from forestry and agriculture products.

A literature search revealed that none of the existing processes are commercially promising. The literature on liquid culture ligninase sometimes conflicts with solid culture results, particularly over nutrient and oxygen responses and whether liquid culture ligninases will completely depolymerize lignin.^{12,13,14,15}

The literature does suggest two principal alternative routes to developing a biological delignification process. The first alternative is a delignification pretreatment process by direct culture of the fungus on the fermentation feedstock. This approach is suggested by studies which evaluated cultures of white rot fungi on plant residue¹⁶ and on wood and kraft wood pulp as a bleaching step.^{17,18} This alternative is referred to throughout this report as direct culture pretreatment. The second alternative would use SSC for culture of white rot fungi

to produce a commercial ligninase preparation. The ligninase preparation would be used to pretreat lignocellulose prior to (or simultaneously with) enzymatic cellulose hydrolysis. The feasibility of this approach is suggested by RTI's work with cellulase production and the studies^{3,4,5} describing production of extracellular ligninase by white rot fungi. This procedure is referred to throughout this report as ligninase pretreatment. A diagram of alternative process and options evaluated in the project is shown in Figure 2 in the next section.

Much of the work was devoted to developing methodologies and analytical procedures as a basis for evaluating the process options. This section will describe laboratory and pilot plant equipment, laboratory processes used in evaluating direct culture and ligninase pretreatment, and analytical procedures.

Laboratory and Pilot Plant Equipment

Three types of experimental solid culture systems were used in the project. These were: the 10-culture test stand with computer monitoring and control developed as part of previous DNRC sponsored work⁹ (Contract RAE 85 1055); pilot reactors constructed as part of the DOE, SBIR phase II cellulase project²; and a new laboratory test stand built for this project. The new test stand was developed without sophisticated monitoring and control systems as a low-cost way to increase capacity. This was necessary because of long culture times, the need to screen

substrates, and the need to evaluate a wide range of variables in direct culture pretreatment processes. The test rack was built to hold 15, 250 cc polycarbonate culture tubes, each with individual manual control for humidified air flow. Two gases, typically air and oxygen, could be blended. Temperature was controlled by an air heater in the enclosed test stand. This was a modification of the proposed design to allow more flexibility in handling cultures. Figure 1 is a photograph of the test stand.

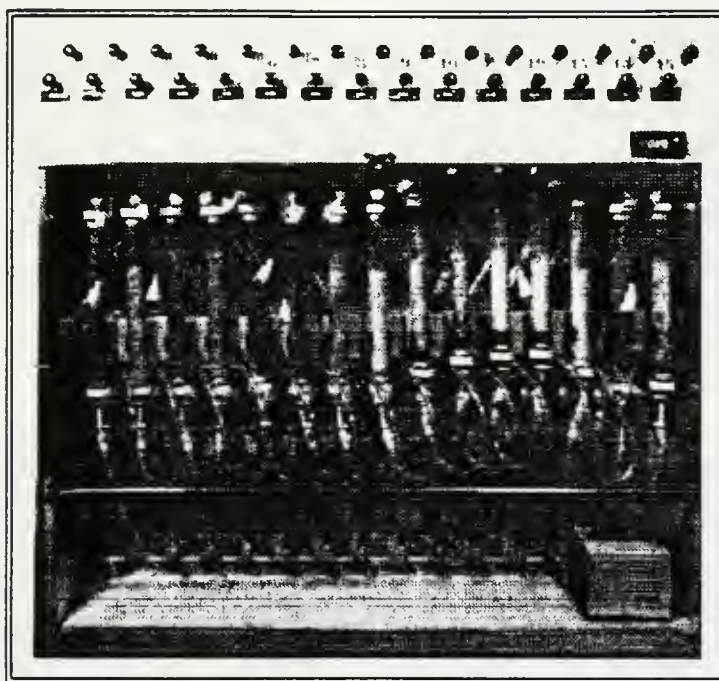


Figure 1. Laboratory SSC test stand.

Pilot reactors were 6-inch X 30-inch glass columns holding about 2 kg of dry weight substrate. Reactors were modified by changes in air systems. In some experiments, reactors were operated horizontally with 500 grams of substrate in a 3-inch deep bed on a tray. In later experiments, columns stood vertically and were filled to about 28 inches deep. Reactors were equipped with compressor or fan driven air systems and humidifiers. Air flow and pressure could be regulated either at the inlet or outlet of the reactor. Monitoring included air flow rate and pressure, temperature, humidity level, and oxygen concentration in exhaust gas.

An air shaker with a capacity of 25, 125 ml flasks or 16, 250 ml flasks was also purchased for the project to increase capacity for hydrolysis and fermentation experiments.

A variety of Mycotech's general laboratory support equipment was used in the project. Key equipment included:

- Blenders
- Centrifuge and filter systems used in enzyme extraction
- Shimadzu 260 recording spectrophotometer used in enzyme and colormetric reducing sugar assays and in evaluating lignin solubilization in pretreatments
- Yellow Springs Instrument Model 23A enzymatic glucose analyzer used in hydrolysis studies
- Varian model 3700 gas chromatograph used for ethanol determinations
- Clean rooms used for maintenance and transfer of white rot fungus cultures

Organisms

Organisms tested in the project were selected from literature review¹⁹ and because they were being used in related contract work. Selection criteria included such factors as known production of different types of ligninases and reports of tests in solid culture. Five organisms were tested in various process alternatives: Phanerochaete chrysosporium,²⁰ Pleurotus ostreatus,¹⁹ Phlebia (Merulius) tremellosus,¹⁷ Trametes versicolor,^{20,21} and Bjerkandera adusta.²² Cultures were obtained from public culture collections and private companies. Cultures were maintained on agar slants with periodic transfer to fresh media. Inoculum cultures were fresh agar slants or liquid cultures in either carbon nitrogen limited salts media or glucose, peptone, yeast extract media. Bjerkandera inoculum cultures were grown with agitation, while other organisms were generally grown in stationary culture.

Analytical and Process Analysis Procedures

Many methods have been used to directly measure lignin biodegradation: release of radiolabeled carbon dioxide from ¹⁴C lignin, high performance chromatography of soluble lignin degradation products, UV absorption scans of soluble lignin degradation products, and determination of substrate lignin content using digestion methods.^{19,20} The common disadvantage of all these methods is difficulty in correlating values of lignin degradation with the bioconversion efficiency of pretreated

lignocellulose feedstocks. As a result, the principal method to evaluate delignification in the project was to measure enzymatic cellulose hydrolysis of pretreated lignocellulose. This is an indirect measure of delignification but does provide a direct measure of the end product of an enzymatic delignification and bioconversion process - namely glucose or ethanol. With hydrolysis conditions held constant, variation in the rate and extent of cellulose hydrolysis is a measure of delignification efficiency.

Hydrolysis was assayed by glucose or total reducing sugar assay. In most cases, glucose assay of hydrolysis was used to evaluate delignification processes. However, in some cases yeast was added to the system and results determined as ethanol. A commercial strain of Saccharomyces cerevisiae (Gist Brocades Fermipan), previously used in cellulose hydrolysis and fermentation, was used in these experiments.

In general, cellulose hydrolysis was carried out using either Mycotech's solid culture cellulase or a commercial cellulase, Genencor 150L, at a dose of 1 percent w/w (0.1 g cellulase per 1 g of dry weight lignocellulose). Hydrolysis was supplemented with a cellobiase preparation (Novo 188) to ensure that soluble cellodextrins were quantitatively converted to glucose. When hydrolysis was carried out as a distinct process step, conditions were pH 4.8 in 0.4 M acetate buffer at 45⁰ C for 24 hours. Conditions were varied when hydrolysis was carried out simultaneously with ligninase treatment and/or fermentation.

Some of the analytical methods employed were specific to one of the two processes - direct culture pretreatment and ligninase pretreatment - evaluated in the project.

Direct culture pretreatment methods

In the direct culture process, white rot fungi were grown on a lignocellulose feedstock. After a suitable culture period the material was washed, suspended in buffer, and tested for cellulose hydrolysis. In some cases, the initial wash was made quantitatively and delignification was evaluated by UV scan (320-190nm) to indicate the level of soluble, UV absorbing, lignin degradation products.

Variables affecting the direct culture process include organism, inoculum, substrate treatment, culture nutrients, time, temperature, and aeration/O₂ enrichment. Lignocellulose feedstocks tested were barley straw, pine and fir sawdust, wood chips (pine), brown paper bag, and two types of wood pulp (kraft process hardwood and softwood pulp from the International Paper Company). Organisms tested were Phanerochaete, Phlebia, Pleurotus and Trametes (these experiments were conducted prior to receipt of Bjerkandera). In general, a liquid culture started from a slant was used as an inoculum, although spores from slant cultures were also employed. Lignocellulose materials were wetted to 60 - 70 percent moisture content with a nutrient solution, autoclaved, inoculated, and transferred to 250 cc columns. Aeration was generally at 25 cc per minute with air or with air supplemented to about 60 percent oxygen. Culture

temperature was 30⁰ C for all organisms except Phanerochaete which was grown at 37⁰ C. Nutrient solutions were varied. A basic salts solution using low levels of inorganic nitrogen was used for nitrogen limited conditions. The nutrient solution was supplemented with varying levels of peptone, yeast extract, and glucose for high nitrogen/carbon conditions. Veratryl alcohol at 0.7 g/l in nutrient solution served as an inducer in some experiments.

Ligninase pretreatment methods

In ligninase pretreatment, white rot fungi were grown under conditions (determined in related contract work) to produce ligninase. These enzyme production cultures were used either as solid, whole-cell preparations or as cell-free liquid extracts in pretreatment of lignocellulose feedstocks. Barley straw milled to pass through a 20-mesh screen (0.850mm) was used in all ligninase pretreatment experiments. In some cases, straw was water washed and dried prior to use.

For whole-cell preparations, whole wet culture was simply mixed as is, at varying levels, in buffer suspensions of barley straw and incubated for 12 hours to 7 days. Tests included whole-cell preparations from Phanerochaete, Trametes, Phlebia, and Bjerkandera.

Cell-free enzyme preparations were made by extracting enzyme production cultures in water or salt solutions followed by centrifuging and filtering through 0.8u polypropylene filters. Two organisms, Trametes and Bjerkandera, were used for cell-free

ligninase preparations. Two ligninase preparations from liquid culture of Phanerochaete were obtained from another company and used for comparison.

Pretreatment reactions with peroxide and manganese dependent ligninase activities were supplemented as appropriate. Reaction buffers were acetate pH 4.5, tartrate pH 3.5, tartrate pH 4.0, malonate pH 3.5, malonate pH 4.0, or phosphate pH 5.7. After treatment, straw was either removed, washed, and resuspended in cellulose hydrolysis buffer, or buffer and cellulase were added directly to the reaction flask. Wash liquid was evaluated by UV scans.

Different enzyme assay procedures were used to evaluate enzyme preparations depending on the organism and type of ligninase activity. Enzyme assays were based on oxidation of lignin model compounds - veratryl alcohol,⁴ phenol red,¹⁹ and anis alcohol.²² Veratryl alcohol and anis alcohol are oxidized to the aldehyde with a corresponding increase in optical density at 310nm for veratryl aldehyde and 285nm for anis aldehyde. In the phenol red assay, oxidation was carried out at acidic pH (3.5 - 5.5). When the reaction is stopped by changing pH to alkaline conditions, the degree of oxidation is determined by a change in absorption of 600nm.

The choices of organism and culture conditions were used to produce four different types of ligninase activities from solid culture:

Peroxidase: Hydrogen peroxide dependent oxidation of phenol red or veratryl alcohol.

Mn Peroxidase: Hydrogen peroxide and manganese dependent oxidation of phenol red or veratryl alcohol.

Mn dependent Oxidase: Manganese dependent oxidation of phenol red.

Oxidase/Laccase: Oxidation of phenol red, veratryl alcohol, or anis alcohol without hydrogen peroxide or manganese.

Each organism produced one or more of the four types of ligninase. However, the different types of enzyme from Trametes and Bjerkandera solid culture and Phanerochaete liquid culture showed significant differences in oxidation of the different lignin model compounds. This indicates that the same type of enzyme (i.e., peroxidase) from the different organisms and culture systems functions differently.

By varying culture conditions, Trametes cultures could be manipulated to selectively produce each of the four types of activities. Trametes enzyme would oxidize phenol red but not veratryl or anis alcohol. Culture extracts were assayed at pH 4.5 in acetate buffer with all combinations of the presence or absence of peroxide and manganese to distinguish the different types of activity. Bjerkandera cultures could be manipulated to selectively produce either a manganese dependent peroxidase or an oxidase. Both enzymes oxidized anis alcohol and veratryl alcohol.

The two Phanerochaete liquid culture preparations were: a peroxidase (not requiring manganese) that oxidized veratryl alcohol but only poorly oxidized phenol red and anis alcohol, and a laccase that oxidized phenol red but no other compounds.

Assays did not distinguish whether the non hydrogen peroxide, non manganese dependent Trametes activity was an oxidase or a laccase. The Bjerkandera enzyme was an oxidase, requiring molecular oxygen for activity and generating H_2O_2 as a reaction product.²² The Phanerochaete liquid culture was described by the donor as a laccase.

These enzyme preparations provided a wide spectrum of ligninase activities. They were tested singly and in various combinations in the pretreatment of barley straw. Four different delignification process options were tested using cell-free ligninase preparations as shown in Figure 2 in the next section. In sequential process steps, reaction conditions of pH, buffer, and temperature could be varied for ligninase treatment and then changed to optimum conditions for cellulase. In simultaneous treatment steps, compromise conditions were employed. Generally, Trametes enzyme was used in acetate buffer pH 4.5 - 5.0, Bjerkandera enzyme in phosphate buffer pH 5.0 - 5.7, and Phanerochaete liquid culture preparations in tartrate pH 3.5 - 4.5. However, a large number of variations were tested. Hydrogen peroxide and manganese were added when using peroxidases.

Controls were run with all experiments. The basic procedure was to run controls without ligninase through the entire pretreatment (duplicating incubation times, different buffers, etc.) and through the hydrolysis procedure being tested. This provided a background hydrolysis level for straw in each treatment variation tested.

RESULTS AND DISCUSSION

The two overall process and the options within each process that were tested during the project are described below and shown in Figure 2.

Description of Process Options

1) Direct Culture Pretreatment

Delignification by direct culture of white rot fungi on lignocellulose feedstock. Two process options for hydrolysis and fermentation of pretreated feedstock were tested.

- A. Sequential cellulose hydrolysis and fermentation in two separate process steps
- B. Simultaneous cellulose hydrolysis and fermentation in a single process step.

2) Ligninase Pretreatment

Delignification by treatment with ligninase enzyme preparations. Fungus is grown using substrates and culture conditions for optimal ligninase production. Six process options were tested.

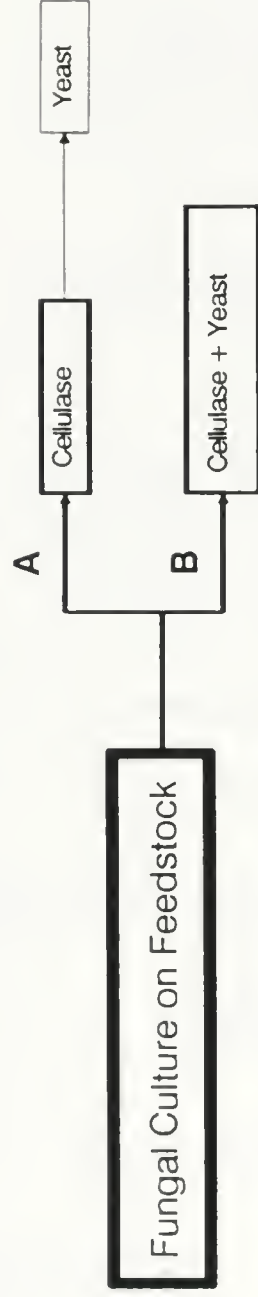
Whole-cell Ligninase: Whole culture used as ligninase preparation.

- A. Sequential ligninase pretreatment and cellulose hydrolysis in two separate process steps.
- B. Simultaneous ligninase pretreatment and cellulose hydrolysis in one process step.

Cell-free Ligninase: Ligninase production culture extracted and filtered to remove cells and culture solids.

- C. Sequential ligninase pretreatment, cellulose hydrolysis, and fermentation in three separate process steps.
- D. Sequential ligninase pretreatment, and simultaneous cellulose hydrolysis and fermentation in two process steps.

1. Direct Culture Pretreatment



2. Ligninase Pretreatment

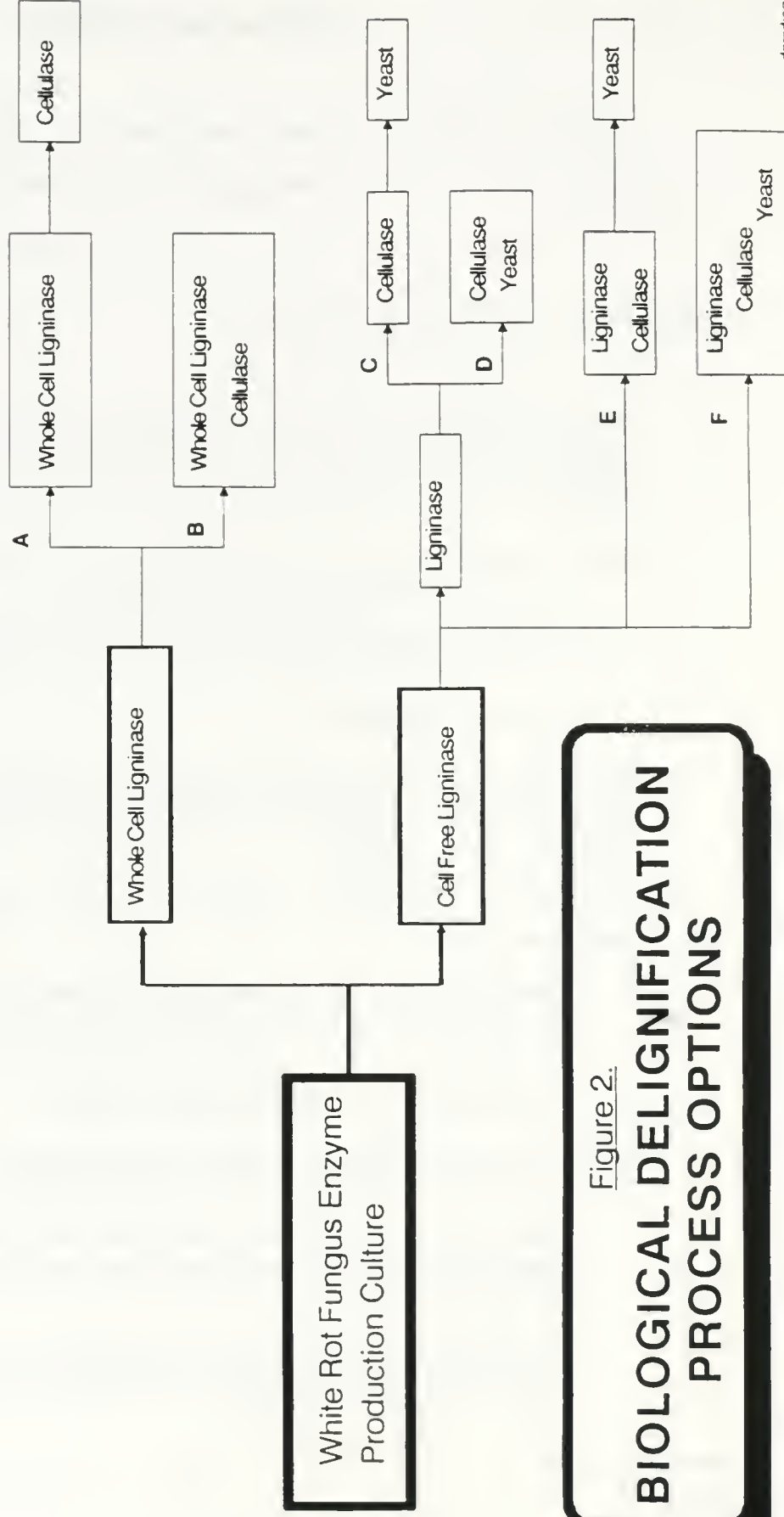


Figure 2.

**BIOLOGICAL DELIGNIFICATION
PROCESS OPTIONS**

- E. Simultaneous ligninase pretreatment and cellulose hydrolysis followed by fermentation in two process steps.
- F. Simultaneous ligninase pretreatment, cellulose hydrolysis, and fermentation in a single process step.

Direct Culture Pretreatment

Direct culture pretreatment experiments evaluated four different fungi grown on seven different representative lignocellulosic materials. Steam-rolled barley was used as a control with known solid culture characteristics.

Best cell growth was obtained using straw with 1 - 5 percent ground paper bag. Very high cell densities were obtained in 7-20 day culture times. Wood pulp also supported good growth, and in samples returned to International Paper, showed reduction in lignin content as measured by brightness and Kappa number (residual lignin content). Fir sawdust showed no growth and pine sawdust minimal growth.

Experiments were repeated using straw as the substrate with variations in culture time, nutrients, oxygen enrichment, and organism. In the majority of experiments, subsequent hydrolysis with cellulase showed no significant difference in glucose yield or extent of hydrolysis compared with untreated controls. In most cases glucose yield was less than in controls. With some Phanerochaete and Pleurotus treated straw, there was about a 10 percent increase in glucose yield. In these experiments, the extent of hydrolysis of untreated straw controls was about 9 percent, and the best level achieved with direct culture

pretreatment was about 10 percent. This compares with about 60 percent hydrolysis obtained with straw chemically delignified by autoclaving in 1 percent NaOH, followed by washing and neutralization.

In treatments showing increased cellulose hydrolysis the amount of glucose produced was too small to be practical. The decreased hydrolysis seen in most experiments was probably due to the use of cellulose by the fungus thereby reducing the cellulose available for subsequent enzymatic hydrolysis. Assays of Pleurotis and Phanerochaete culture showed cellulase activity. The cellulase concentration was probably sufficient to hydrolyze readily accessible cellulose in the straw with the resulting glucose used by the fungus.

Several experiments were conducted in which glucose or starch was added to the cultures in an attempt to repress cellulase production. No repression was found. Enrichment of the culture atmosphere up to 100 percent oxygen had no effect on subsequent hydrolysis results.

Water extracts of culture material did show much higher levels of soluble lignin than controls as shown by UV absorption. Often straw would be visibly lighter in color after direct culture pretreatment. Both of these results indicated extensive delignification of the straw; however, there was never a significant increase in cellulose hydrolysis.

Ligninase Pretreatment

Pretreatment with a ligninase preparation has a wider range of process options than direct culture as shown in Figure 2. In this process, the fungus is grown under conditions for optimal production of specific ligninase enzymes. The whole culture may be used as an enzyme preparation, or the ligninase can be extracted to produce a cell-free liquid enzyme preparation. A whole-cell preparation might be the best option for on-site enzyme production and biomass utilization, while a cell-free enzyme could be produced and marketed from a central location. Whole-cell preparations could contain enzyme activities bound to culture substrate that would be lost in extraction. Cell-free enzyme preparations may not contain inhibitors or undesirable activities that could be present in whole-cell culture preparations. With each type of enzyme preparation, the enzymatic ligninase and cellulase reactions and fermentation steps were tested both sequentially and simultaneously. Sequential processes have the advantage that each reaction can be run at optimal conditions. Simultaneous processes have the advantage of simplicity in process design and equipment.

Whole-cell enzyme preparations were evaluated in sequential and simultaneous delignification and cellulase hydrolysis. Cell-free enzyme preparations were tested in both sequential and simultaneous enzyme steps and in enzyme steps coupled with fermentations. Fermentations were run as a third sequential

step, simultaneously with the cellulase hydrolysis step and simultaneously with both enzyme reactions.

A number of variables were evaluated for each step in each of the process options. These included:

- Straw concentration
- Ligninase source organism
- Ligninase type (peroxidase, oxidase, or mixtures)
- Ligninase concentration and reaction time
- Buffer system and pH
- Control of bacterial and fungal growth
- Cellulase preparation and concentration
- Fermentation conditions

Whole-cell ligninase

In experiments with whole-cell ligninase preparations, straw concentration was a slurry of 10 percent straw or a moist solid at 30 percent straw. In sequential cellulose hydrolysis, straw concentration was 5 percent after all additions of buffer and enzyme. Trametes preparations were cultures with peroxidase, manganese-dependent peroxidase, or oxidase activities.

Bjerkandera preparations contained manganese dependent peroxidase or laccase, and Phlebia contained peroxidase. Trametes and Bjerkandera enzymes were tested singly and in numerous combinations. Liquid culture Phanerochaete peroxidase and laccase preparations were added to whole-cell preparations in some experiments. Enzyme concentrations of 10 - 50 percent of the weight of straw were tested with reactor times of 12 hours to seven days. Acetate and tartrate buffers and unbuffered systems were tested. Straw was used either sterilized in buffer or unsterilized with ammonium bifluoride or antibiotics added in

some cases to suppress bacterial growth. Cellulase was a commercial preparation from Genencor.

Results showed delignification with solid culture Trametes and liquid culture Phanaerochaete preparations as measured by UV absorption of lignin degradation products in wash water from treated straw. Inactivated whole culture ligninase preparations were used in these experiments to control for lignin degradation products introduced with the crude enzyme preparations. In some cases, washed treated straw was visibly lighter in color. These indications of delignification did not correlate with significant increases in cellulose hydrolysis. Best results were about 10 percent conversion of total straw weight compared with about 8 - 9 percent in controls. Both the extent of conversion and relative increase compared with controls were not significant.

Cell-free ligninase

Cell-free enzyme preparations were tested extensively using solid culture extracts with peroxidase and oxidase from Trametes and Bjerkandera and the liquid Phanerochaete preparations. Ligninase preparations were used singly and in all possible combinations in both sequential and simultaneous cellulase hydrolyses.

An extensive set of tests were conducted to follow stability of ligninase in these reactions. The first experiments evaluated binding to straw. With this baseline, delignification reactions were sampled and assayed for enzyme activity over time. All enzyme preparations showed some binding to straw. Stability

assays showed that Trametes enzyme could not be recovered from reaction mixtures after a few hours. This activity, particularly the oxidase, is either unstable or becomes irreversibly adsorbed to straw. Both Bjerkandera and Phanerochaete enzymes could be recovered even after three days of reaction, indicating good stability and equilibrium binding conditions.

A variety of buffer systems and pH profiles were analyzed. Optimum conditions for each ligninase were used for single enzyme reactions. In sequential processes with mixed ligninase preparations and in simultaneous cellulase hydrolysis, compromise conditions were tested. Enzyme assays were run on each enzyme preparation and type of ligninase activity to establish buffer and pH range for activity. In mixed ligninase systems, conditions were found in which all components of the mixture showed at least a significant fraction of optimal activity. Cellulase preparations have a pH optimum of about 4.8; however, the activity is retained over a broad pH range. Therefore, conditions favorable for ligninase activity could be employed in simultaneous ligninase/cellulase systems.

Reaction times from 12 hours to seven days were run in different experiments with liquid ligninase concentrations as high as 50 percent by volume of reaction mixtures.

In experiments with long culture times, it was necessary to control bacterial contamination and fungal growth from ligninase and cellulase preparations. A series of experiments were conducted to test the effects of several different chemical and

antibiotic control agents on ligninase and cellulase activities. Combinations were found that did not affect enzyme activity and maintained uncontaminated reactions for up to a week.

Three different cellulases were employed in these experiments. The liquid, commercial cellulase is high in specific (endo- and exo-) cellulase activities, but very low in "side" activities, particularly hemicellulose degrading xylanase. Reasoning that hemicellulose degradation may be important in pretreatment, we tested two cellulase preparations with high levels of xylanase. These were a preparation from Amano and Mycotech solid culture cellulase preparations.

Fermentations were run using buffer and bacterial suppression that did not affect yeast. Fermentation controls were run using hydroxide delignified straw in addition to straw without ligninase treatment.

Results of experiments with cell-free ligninases (extracts) were similar to other process experiments. Straw showed evidence of delignification, but economical levels of conversion to glucose or ethanol were not obtained. Best results were obtained in experiments using combinations of peroxidase and oxidase type ligninase activities as a pretreatment followed by simultaneous hydrolysis and fermentation of the cellulose. (Figure 2, option D.) In some experiments, alcohol concentrations of 0.26 - 0.37 mg/ml were obtained compared with 0.15 - 0.16 mg/ml in controls. Although twice the alcohol concentration was obtained, overall conversion of straw was only about 10 - 12 percent. This

compares poorly with 60 percent overall conversion of straw that is hydroxide pretreated and hydrolyzed.

OVERALL PROCESS DESCRIPTION OF LIGNOCELLULOSE TO ETHANOL

The overall description of alternative processes for conversion of lignocellulose to ethanol was shown in Figure 2 in the previous section. None of the process alternatives we tested showed economically feasible conversion rates. The best results were obtained with delignification using a mixed cell-free ligninase enzyme preparation followed by simultaneous cellulase hydrolysis and fermentation. (Process option D, Figure 2.) A schematic of this type of process is shown in Figure 3.

An overall mass and energy balance can not be established from the available data. Overall conversion was too low to establish ligninase dose response or lignin removal. Alcohol concentrations in fermentations were only about 0.2 - 0.4 mg/ml which is too low to economically distill without a concentration step. Evaluation of the mass and energy balance of a dilute beer concentration step were beyond the scope of this project.

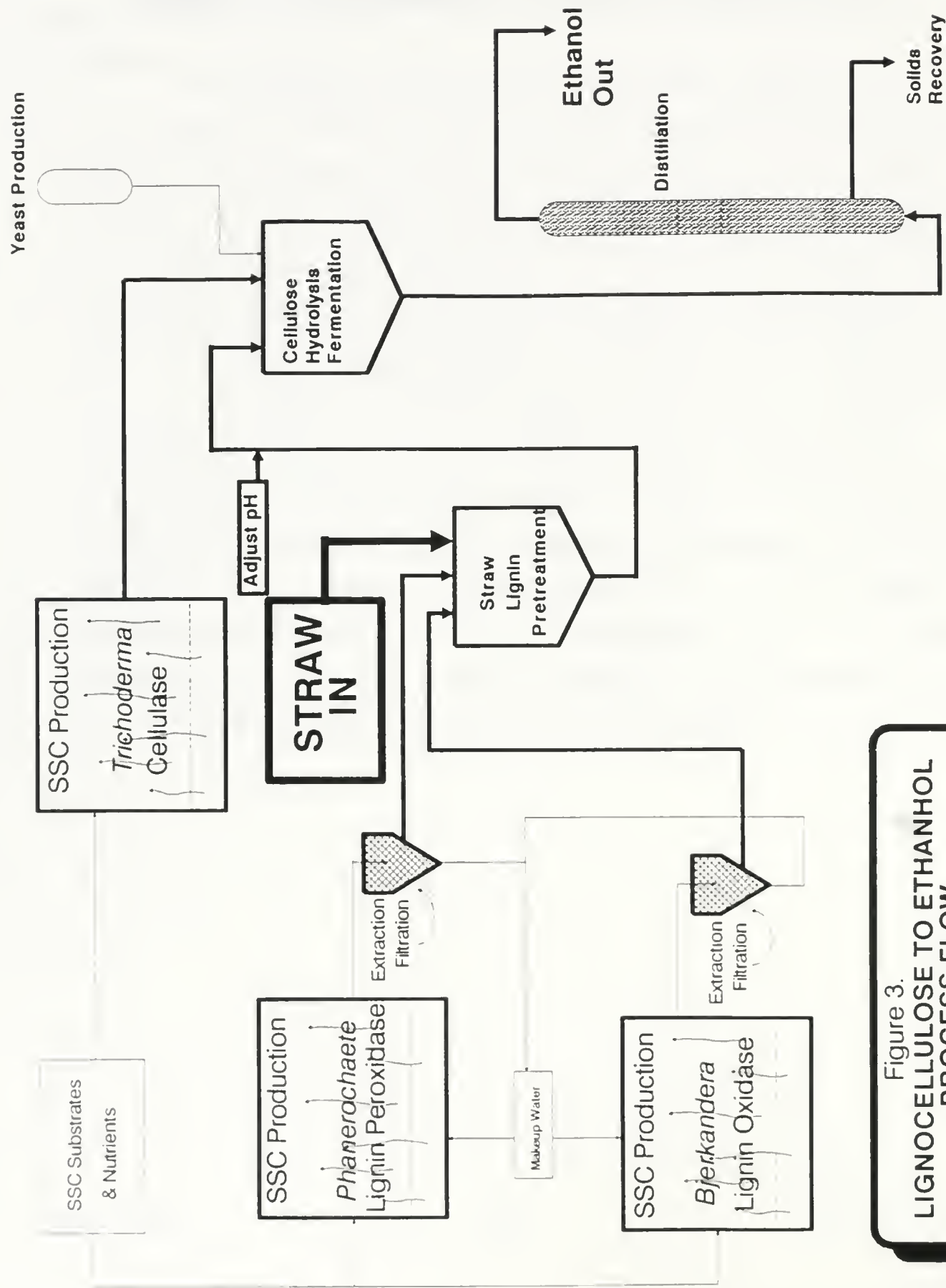


Figure 3.
LIGNOCELLULOSE TO ETHANOL
PROCESS FLOW
DIAGRAM

PRELIMINARY ECONOMICS

Best results with enzymatic delignification showed about a 10 percent overall conversion of straw to ethanol on a weight basis. The alcohol yield and value for conversion of one ton of straw is shown below.

2000 lbs straw X 0.10 conversion = 200 lbs glucose

200 lbs glucose X 0.51 theoretical molar fermentation efficiency = 102 lbs ethanol

102 lbs ethanol ÷ 6.6 lbs ethanol per gallon = 15.4 gallons

15.4 gallons X \$1.65/gallon selling price for ethanol = \$25.41.

In southwest Montana, the cost for straw collected and delivered to a central point is in the range of \$25 - \$30 a ton. Conversion efficiencies obtained in the project would not cover feedstock cost for alcohol production. Results did not justify further in-depth economic analysis.

RESULTS AND CONCLUSIONS

The project did not demonstrate economic feasibility of a completely biological conversion of lignocellulose to ethanol. Results consistently showed increased cellulose hydrolysis of ligninase pretreated straw compared with controls; however, the increase fell far short of conversion efficiencies necessary for economic ethanol production.

Clearly, straw was delignified, but the question remains: why did this not correlate with increased cellulase hydrolysis. The extent of delignification may have been insufficient to expose a significant fraction of the cellulose for enzymatic hydrolysis. Alternatively, there may be other factors critical to delignification in which the enzymatic ligninase and cellulase preparations were not effective. These could include secondary structures such as linkages between cellulose, hemicellulose, and lignin, or tertiary structure limiting access of the enzyme.

Delignification might be increased by the use of higher enzyme concentrations, longer reaction times, or different types of activities. Economics of these steps would be questionable as the project tested a very wide range of ligninase sources, concentrations, and long reaction times without achieving good yields.

There may be some promise in testing a pretreatment that combines enzymatic, chemical, and mechanical processes. The cost and efficiency of chemical/mechanical delignification might be

improved sufficiently by an enzymatic step to justify enzyme cost. A mild chemical treatment to disrupt secondary structure, or milling to disrupt tertiary structure, could improve enzymatic delignification enough for a conversion process to be economical. If further research were to be conducted based on this project, a combination of mechanical, chemical, and enzymatic pretreatment is one recommended approach.

One other important factor may have been the choice of lignocellulose feedstock. Barley straw was the primary feedstock for this project because it is low in cost and readily available in southwest Montana. Other feedstocks, such as, aspen, poplar, or other agricultural residues, may be more amenable to enzymatic pretreatment. This is supported by recent work in Canada²⁵ using aspen wood delignified by direct culture treatment with Phlebia tremellosus. After a 12 week culture time, cellulose hydrolysis and fermentation of delignified aspen wood yielded 0.116 g ethanol per g of wood - an overall conversion efficiency of about 23 percent of theoretical. There may also be partially delignified waste material such as waste paper in which an enzymatic delignification would be a cost effective conversion step. These types of alternatives were too wide ranging to be effectively addressed in this project.

In conclusion, the project did make a significant, if negative, contribution to work in this field. Because of integration with two other projects related to white rot fungi, Mycotech was able to test a large number of different enzyme

preparations under a very broad range of conditions. A comprehensive evaluation of different types of ligninases from five different fungi does provide a good basis for further work, and probably indicates that factors other than ligninase need to be addressed in future work on enzymatic pretreatment.

APPENDICES

1) Work Schedule

The time line of the original contract was extended considerably from April 30, 1988 to September 30, 1989. The principal reason for this was that initial experiments with the direct culture process route were unsuccessful and efforts were focused on using ligninase preparations. Mycotech received two other projects that included work to produce and evaluate ligninase enzyme preparations from different organisms. Extending the DNRC project provided a unique opportunity to test enzyme produced for these other projects in the DNRC biomass conversion project. The increased time allowed testing of a much wider range of organisms and enzyme preparations, and to have more completely characterized these enzyme preparations, than would have been possible within the scope of the DNRC project alone.

2) Budget

Budgeted and actual expenses by project milestone are shown below. The major changes in budgeted vs. actual expenses were in subcontracts and associated travel expenses. Results did not justify subcontractor tests of ligninase preparations. As a result, the subcontract and the supporting travel budget were transferred to salaries and indirect costs for additional experimental work on alternative process options and enzyme preparations.

Budget Summary

	<u>Budgeted</u>	<u>Actual</u>	<u>Billed</u>
MS1	16556.50	15608.45	15608.45
MS2	9033.46	8633.46	8633.46
MS3	10589.55	11011.40	11011.40
MS4	13895.79	13895.75	13895.79
MS5	12593.05	13512.46	13512.46
MS5A	13268.64	13269.64	13269.64
MS5B	14887.20	8237.20	8327.20
Sub Total	<u>90825.19</u>	<u>84168.40</u>	<u>84168.40</u>
MS6			
Salaries	3040.00	7090.00	
Fringe Benefits	1051.84	2453.14	
Travel	650.00	0.00	
Misc. & Ind.	3191.47	7443.65	
Sub Total	<u>7933.31</u>	<u>16986.79</u>	<u>14590.00</u>
Project Total	<u>98758.50</u>	<u>101155.19</u>	<u>98758.50</u>
In-Kind Contribution			2396.69

Two other projects on production of ligninase by white rot fungi provided enzyme preparations for tests of straw conversion in the DNRC project. An exact matching value cannot be determined as the other projects did not have biomass conversion as a direct project objective. However, the production and evaluation of cell-free enzyme preparations from these projects was a considerable contribution. Total amounts of related white rot fungus contracts were:

US Dept of Energy SBIR Phase I	\$ 49,780
Private Company Contract	219,500
	\$269,280

3) Technology Transfer/Commercialization Activities

The project did not demonstrate an economically feasible process for enzymatic delignification of Montana sources of lignocellulose. As a result, Mycotech has not initiated any commercialization activities from this project.

The original work plan included provisions for tests to be subcontracted to SERI (or TVA) using Mycotech ligninase preparations. Because results did not show significant pretreatment, the outside tests were dropped in order to conduct additional tests of alternative process options in house. Because results did not show significant conversion efficiencies, and SERI was not involved in tests, the report was not sent for outside review.

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